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Proposed temperature-dependent conformational transition in D-amino acid oxidase: A differential scanning microcalorimetric study

(fluorimetry/nonlinear Arrhenius plots/transition enthalpy/two-state processes/van't Hoff enthalpy)

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ABSTRACT **A number of authors have reported observations on D-amino acid oxidase [D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3] that they have interpreted in terms of a temperature-dependent conformational transition having a van't Hoff enthalpy amounting to more than 1 cal per g of protein (1 cal = 4.184J). No indication of this transition is obtained by using a differential scanning calorimeter having a sensitivity considerably in excess of that required to detect such a transition. The implications of this discrepancy are discussed.**

Massey *et al.* (1) reported temperature dependencies for several properties of D-amino acid oxidase [DAAO, D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3] that they interpreted in terms of a thermally induced conformational transition in the enzyme. The most significant of these for our present purposes was a 30% decrease in the tryptophan fluorescence of the enzyme as the temperature was raised from 8° to 22°. This decrease, which was reversible, followed the symmetrical sigmoidal curve expected for a two-state process with an enthalpy value, ΔH_{vH}, derived from the van't Hoff equation equal to 78 kcal mol^{-1}. Additional observations by Massey *et al.* (1), as well as by other authors, have been cited as further evidence for the existence of this transition (see *Discussion*).

The most direct means for observing a thermally induced change in a protein is differential scanning calorimetry (2). For any two-state equilibrium process not involving intermolecular cooperation—such as was assumed for the purported transition in DAAO in deriving a value for ΔH_{vH} from the data on the fluorescence quenching—the apparent enthalpy, ΔH_{vH}, must be equal to the actual enthalpy, ΔH_{cal}, as measured in the scanning calorimeter. Although no general proof has been given, a consideration of simple model systems (3) suggests that for any process more complicated than the two-state process,† $\Delta H_{cal} > \Delta H_{vH}$. We assume that in *all* cases *not* involving *intermolecular* cooperation

$$\Delta H_{cal} \geq \Delta H_{vH} \qquad [1]$$

A process having ΔH_{vH} = 78 kcal mol^{-1}, so that $\Delta H_{cal} \geq 78$ kcal mol^{-1}, that occurs with a protein of molecular weight as low as that of DAAO should be readily detectable in a scanning calorimeter of high sensitivity such as that described by Privalov *et al.* (4). In this paper we report the results of a study of the thermal behavior of DAAO using the Privalov calorimeter.

MATERIALS AND METHODS

Hog kidney crystalline DAAO (suspension in ammonium sulfate), thymol-free catalase, and FAD disodium salt, grade III, were purchased from Sigma Chemical Co., St. Louis, MO, and were used without further purification. All other chemicals employed were of analytical grade. Doubly deionized water was used throughout. All experiments were carried out in 0.05 M sodium pyrophosphate buffer, pH 8.5.

Details concerning the preparation and analysis of holo- and apoenzyme solutions and those concerning activity determinations were described in a previous publication (5). The appropriate dialysates were used as reference solutions in the differential scanning calorimetry experiments. It was ascertained that no significant losses of enzyme activity were incurred in any experiments, except for fluorescence measurements on the apoenzyme in which photodegradation was a problem.

Most of the calorimetric experiments were performed with a Privalov calorimeter (4) purchased through Mashpriborintorg, Moscow, U.S.S.R. This instrument has a sample volume of 1 ml, and, at the scan rate used in all experiments (1 K min^{-1}), it has a sensitivity of approximately 20 μcal K^{-1} ml^{-1}. Preliminary experiments were run in a somewhat less sensitive instrument (3). The results obtained with the two instruments agreed well. Those reported here were obtained with the Privalov calorimeter.

The densities of the protein and buffer solutions were determined in a vibrating tube densimeter, model 01D, Sodev Inc., Sherbrooke, Quebec, Canada, at 3°–5° intervals. The densimeter was calibrated with nitrogen and water before and after each density measurement.

Fluorescence measurements were made with a system composed of a mercury–xenon arc source, two Bausch and Lomb monochromators, a temperature regulated cell holder, and a photomultiplier detector amplifier. Excitation and emission were at 295 and 333 nm, respectively, for the holoenzyme and at 295 and 340 nm for the apoenzyme. Absorbances at the exciting wavelength were determined in the same apparatus and never exceeded 0.1.

Temperatures were controlled and measured in all experiments to within ±0.1°.

RESULTS

Fluorescence measurements

We have checked the observation of Massey *et al.* (1) that the fluorescence yield of the holoenzyme of DAAO decreases by

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Abbreviation: DAAO, D-amino acid oxidase (EC 1.4.3.3).

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† Although, as mentioned, the van't Hoff relation is valid only for a simple two-state process, it is still possible to obtain a value for ΔH_{vH}, which will in general vary with temperature, for more complex processes from the slope of the ln K *vs* $1/T$ plot, where K is the equilibrium constant and T is the absolute temperature.

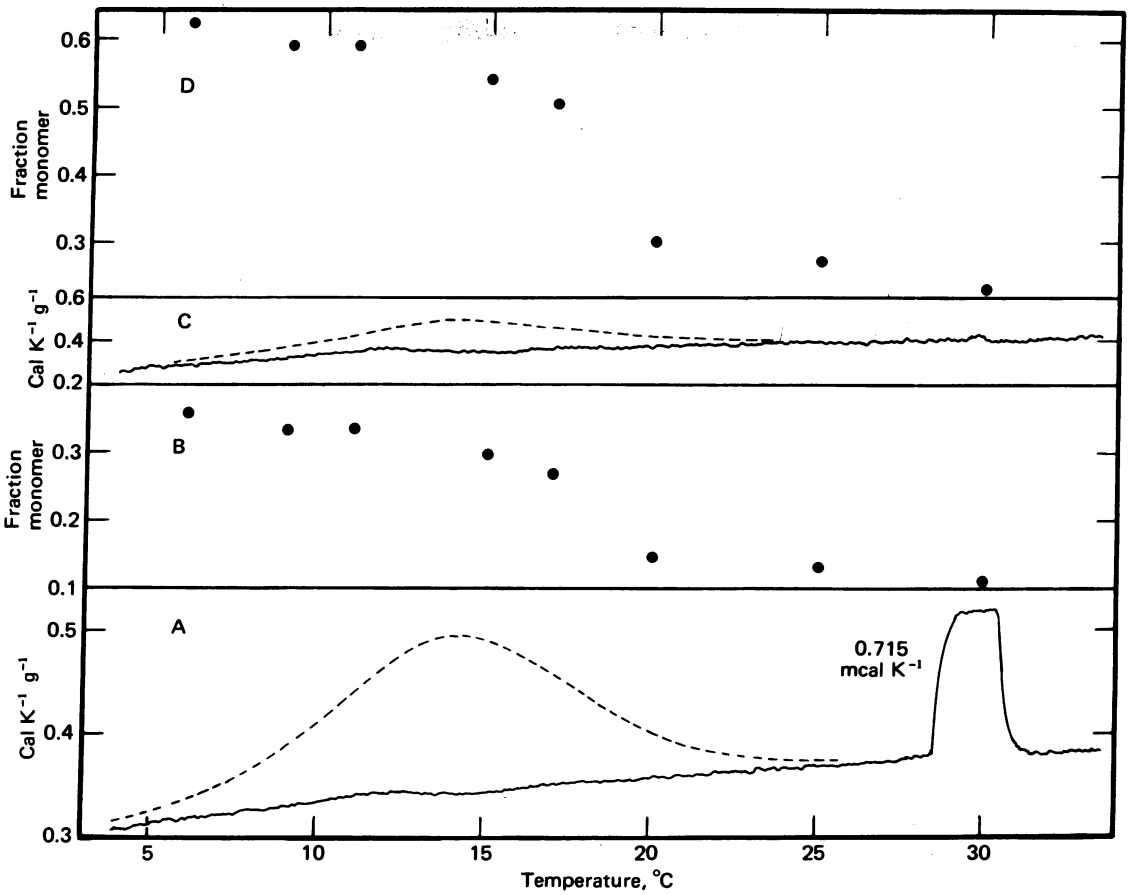

FIG. 1. (*A*) Solid line: variation with temperature of the apparent specific heat of DAAO at a concentration of 5.03 mg ml^{-1}; the peak at about 30° is due to calibration heat supplied to the reference cell. Dashed line: expected curve for a conformational transition with enthalpy absorption of 51 kcal mol^{-1}. (*B*) The fraction of the enzyme in monomeric form calculated by using the equilibrium constant for dimerization reported by Shiga and Shiga (7). (*C* and *D*) Similar to *A* and *B*, respectively, for enzyme at a concentration of 0.98 mg ml^{-1}.

approximately 30% in the temperature range 0°–25° in a completely reversible manner. For enzyme concentrations from 0.65 to 2.15 mg ml^{-1}, we have found the mid-temperature, T_m, of the decrease to vary between 12° and 13°, and the van't Hoff enthalpy, ΔH_{vH}, to vary between 50 and 54 kcal mol^{-1} with no discernible, dependence on concentration. The value for ΔH_{vH} reported by Massey *et al.* (1) is approximately 50% larger than we have found. The source of this discrepancy is unknown.

The apoenzyme also shows a decrease in fluorescence emission in this temperature range. In this case, photodegradation of the protein precluded our obtaining accurate results. The approximate values observed were T_m = 14° and ΔH_{vH} = 32 kcal mol^{-1}.

Estimation of the quantum yields for the fluorescence of both proteins at 25°, by comparison with two proteins and three tryptophan derivatives of known quantum yield, gave 0.09 ± 0.02 for the apoenzyme and 0.06 ± 0.02 for the holoenzyme. These are reasonable values for a protein and indicate that the observed fluorescence is an intrinsic property of the proteins and is not due to an impurity.

Calorimetric results

In no calorimetric scan was there any indication of excess absorption of enthalpy within the temperature range of interest. Typical experiments with the holoenzyme are shown in Fig. 1. The solid line in Fig. 1*A* is a scan observed at a concentration of 5.03 mg ml^{-1}, and includes a 50 μW calibration mark. The slope of this line, equal to approximately 0.003 cal K^{-2} g^{-1}, is similar in magnitude to the gradual increase in apparent specific heat observed with other proteins (6). The dashed line in Fig. 1*A* is the excess specific heat that would have been observed had a two-state process with T_m = 14° and ΔH_{vH} = ΔH_{cal} = 51 kcal mol^{-1} taken place. In Fig. 1*B* the fraction of the enzyme in the monomeric form, as calculated by using the equilibrium constants reported by Shiga and Shiga (7), is given. Fig. 1*C* records the scan observed at an enzyme concentration of 0.98 mg ml^{-1}, and again the dashed curve shows the behavior to be expected if a two-state conformational change had taken place. Comparison of the fractional monomer contents in Fig. 1 *B* and *D* shows that doubling the fraction of monomer does not bring out any transition. We therefore conclude that our failure to observe a transition cannot be attributed to the occurrence of the transition in only one of the two forms, monomer or dimer.

Calorimetric experiments with the apoenzyme likewise gave no indication of a conformational transition.

Density data

In both of the calorimeters employed in this work the cells are completely filled with solvent or solution and are open to a gas phase. Thus any density change taking place during the purported transition would be reflected in a change in the apparent specific heat of the protein. We therefore determined the temperature variation in the densities of the buffer and of solutions of the apo- and holoenzymes. The densities of all three solutions decreased by 0.4% in the temperature interval 6°–27°. They decreased in a strictly parallel manner, so that no de-

tectable contribution to the output signal of the calorimeter could have resulted from this effect.

DISCUSSION

Thermally induced conformational transitions have been proposed frequently in the past and have been accepted as explanations for the temperature dependence of a number of macromolecular properties, particularly in cases in which this dependence followed a sigmoidal course. It is therefore important to consider carefully the apparent discrepancies raised by the fact that differential scanning calorimetry, the most direct means for detecting a two-state change in a macromolecular system, fails to give any indication of a conformational transition in the case of a protein for which the results obtained with several independent indirect observational techniques have led to the postulation of such a change.

Evidence for a Conformational Transition. (*i*) *Fluorescence Changes.* Important evidence pointing toward a temperature-dependent conformational transition in DAAO is the fluorescence observations of Massey *et al.* (1) outlined above. Our fluorescence results are in general agreement with theirs. A conformational transition that increases the exposure of tryptophan side chains to the aqueous solvent would decrease the quantum yield for fluorescence. A conformational transition could also lead to a diminution of internal energy transfer from absorbing chormophores to one or more fluorescing tryptophans.

If the decrease of fluorescence intensity with increasing temperature were the result of a *kinetically* limited process, it would be quite possible to have a large activation energy with a small overall enthalpy change. However, in this case the fluorescence change would not appear as a reversible process.

The discrepancy between the calorimetric and fluorometric data cannot be removed by assuming that there is cancellation of an endothermic transition (evidenced by the fluorescence change) by a coupled or independent exothermic process. Consider, for example, the scheme

$$\text{Step 1: A} \rightleftarrows \text{B}, \quad \Delta H_1 = 50 \text{ kcal mol}^{-1}, \quad K_1 = 1 \text{ at } T_{m1}$$

$$\text{Step 2: B} \rightleftarrows \text{C}, \quad \Delta H_2 = -50 \text{ kcal mol}^{-1}, \quad K_2 = 1 \text{ at } T_{m2}$$

with the three cases (a) $T_{m1} = 15°$, $T_{m2} = 0°$; (b) $T_{m1} = T_{m2} = 15°$; and (c) $T_{m1} = 15°$, $T_{m2} = 30°$. It can be shown by direct calculation that in each case the overall process approximates a two-state process in which (a) A is converted to C with $T_m \approx 7.3°$; (b) equal concentrations of A and C are converted to B with $T_m \approx 17°$; and (c) B is converted to C with $T_m \approx 30°$.

We propose the following as a possible resolution of the discrepancy.‡ It is well known that the fluorescence emission from a macromolecule can be critically dependent on such factors as exposure of fluorescent groups to the aqueous solvent and transfer of intramolecular energy between absorbing and emitting chromophores. It is therefore conceivable that a significant change in fluorescence emission might result from very minor changes in conformation, amounting to a redistribution among the microstates making up a macrostate, which is the native state of the molecule. It seems quite possible, for example, that the gradual thermal expansion of the molecule over a narrow temperature range could permit markedly increased access of solvent molecules to a tryptophan residue. Or, if there were a chromophore that at low temperature is held in an orientation particularly favorable for a dipole–dipole energy transfer that contributes to the fluorescence of the molecule, thermal expansion could result in a sudden increase in the freedom of motion of the chromophore with an attendant decrease in fluorescence emission.

If the above proposal is tenable, it obviously is of general significance in connection with the interpretation of the temperature variation of at least the optical properties of macromolecules.

(*ii*) *Kinetic Data.* Massey *et al.* (1) observed that an Arrhenius plot (log rate *vs* $1/T$) of the enzymic activity of DAAO is nonlinear and can be represented as two straight lines intersecting at 14°. Tu and McCormick (8) found that an Arrhenius plot of the rate of photoinactivation of DAAO could similarly be interpreted as composed of two straight line segments intersecting at an angle of 15°. In both instances the data were taken to indicate a temperature-dependent transition between two forms of the enzyme, with the higher temperature forms having a smaller activation energy than that of the lower temperature form.

We consider this type of evidence to constitute even less positive support for the existence of a transition than the fluorometric evidence, because in each case the data can be fitted within experimental uncertainty to a curved Arrhenius plot with a nonvanishing value for the heat capacity change on activation, $\Delta Cp^{\ddagger}$. In the case of the data of Massey *et al.* (1), the required value of $\Delta Cp^{\ddagger}$ is about -200 cal K^{-1} mol^{-1}. This value is not overly large for a macromolecular system; it is, for example, less than half the total heat capacity change, -530 cal K^{-1} mol^{-1}, that accompanies the binding of the coenzyme, FAD, to the apoenzyme (5). It seems reasonable to expect that values for $\Delta Cp^{\ddagger}$ of this general magnitude will be more frequently encountered in macromolecular systems than will be the unique value $\Delta Cp^{\ddagger} = 0$.

In disagreement with Massey *et al.* (1) Koster and Veeger (9) concluded from their rate measurements with the holoenzyme that there is a temperature-dependent equilibrium between two forms having equal activation energies but, at any given temperature, different activities. They deduce from their data that the enthalpy of transition between the two forms is 55 kcal mol^{-1} and that the equilibrium constant is equal to unity at about 17°.

(*iii*) *Polymerization Equilibria.* Several authors, using various methods of observation, have concluded that the apoenzyme and holoenzyme show temperature-dependent polymerization equilibria (7, 10, 11). A study of this phenomenon in the case of the apoenzyme was made by Henn and Ackers (11) using molecular sieve chromatography. At the concentration level of their experiments, only the dimerization equilibrium need be considered. Because the apparent dimerization constant was independent of temperature below about 12° and above about 15°, they interpreted their results in terms of a temperature-dependent isomerization of the protein, with the low-temperature form having a smaller dimerization constant than the high-temperature form. This interpretation leads to a value of about 200 kcal mol^{-1} for the enthalpy change in the isomerization reaction (G. K. Ackers, personal communication), which would be readily detectable in the Privalov calorimeter. We cannot account for this discrepancy.

CONCLUSION

Further discussion of the experimental observations that have been interpreted in terms of temperature-dependent transitions

‡ This proposal has resulted from discussions with Dr. Meyer Jackson.

in both apo- and holo-DAAO is unnecessary to make it evident that there are serious discrepancies with the results of scanning calorimetry. Although we are unable, in some instances, to resolve these discrepancies, we are sufficiently confident of the calorimetric observations and of their significance to believe that it is important to point out the existence of these discrepancies and to invite further discussion. It may be, for example, that model systems can be devised that will cast doubt on the general validity of Eq. 1.

It is of interest to note here a case in which fluorescence and differential scanning calorimetry data are in at least qualitative agreement. Kelly and von Hippel (12) observed that the fluorescence emission of the gene 32 protein isolated from *Escherichia coli* cells infected with T4 bacteriophage undergoes a decrease in the temperature range 50°–55°. L. Sillerud and K. Williams (personal communication) have found, using the Privalov calorimeter, a transition in this protein in the same temperature region.

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